

with the stimulating effect of SUMO on spontaneous mutagenesis, we found a slightly increased rate (1.3-fold) in the *ulp1*¹² isopeptidase mutant^{6,7}, which displays a partial defect in SUMO deconjugation of PCNA at the permissive temperature (see Supplementary Fig. 2).

Our findings lead us to propose a model for the interplay between ubiquitin and SUMO conjugation during DNA replication and repair (Fig. 3): according to ref. 6, PCNA acts as a molecular switch that in its SUMO-modified form may promote replication, whereas multi-ubiquitination stimulates error-free repair. We have now shown that mono-ubiquitination of PCNA is essential for translesion DNA synthesis by Pol η and Pol ζ, resulting in the accumulation of damage-induced mutations. In addition, Pol ζ is also involved in spontaneous mutagenesis during DNA replication. For this purpose Pol ζ can be stimulated by SUMO modification of PCNA; however, in this replicative mode its activity is not inducible by DNA damage, thus explaining the puzzling mutagenesis phenotypes of *rad6* and *rad18* strains. Our model involving differential activation of Pol ζ by ubiquitinated and SUMO-modified PCNA for mutagenesis is consistent with a twofold origin of mutations: according to current beliefs, spontaneous mutations arise not only from replication across unrepaired lesions^{8,9}, but also from Pol ζ-dependent extension of terminal mismatches, hairpins or other structural features of template DNA difficult to overcome by purely replicative polymerases¹⁰. Thus, we postulate that one function of SUMO during normal S phase is to harness Pol ζ to overcome fork blocks not caused by damage but by other refractory DNA structures. Activation of the repair polymerases by PCNA could be explained by a preferential interaction with the modified forms of PCNA. However, as unmodified, recombinant PCNA interacts productively with Pol η *in vitro*¹¹ and we find that even the PCNA lysine mutants are not impaired in their interaction with Pol η (not shown), we consider it more likely that ubiquitin and SUMO may be responsible for the dissociation of other PCNA-binding proteins, such as replicative polymerases¹², to allow access of the translesion polymerases to the primer terminus. Alternatively, the modifications might not affect binding of the polymerases at all, but could exert a more subtle, modulating effect on their activity or processivity. Future studies will have to address the molecular mechanisms that regulate the balance between SUMO modification, mono-ubiquitination and multi-ubiquitination of PCNA and thereby control the extent of DNA repair and replication. □

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- Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–479 (1998).
- Hicks, L. Getting down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* **9**, 107–112 (1999).
- Conaway, R. C., Brower, C. M. & Conaway, J. W. Emerging roles of ubiquitin in transcription regulation. *Science* **296**, 1254–1258 (2002).
- Urich, H. D. Degradation or maintenance: actions of the ubiquitin system on eukaryotic chromatin. *Eukaryot. Cell* **1**, 1–10 (2002).
- Melchior, F. Non-canonical ubiquitin. *Annu. Rev. Cell. Biol.* **16**, 591–626 (2000).
- Hooge, C., Flández, B., Moldovan, G. L., Pirojwski, G. & Jentsch, S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135–141 (2002).
- Broomfield, S., Chow, B. L. & Xiao, W. MMS2, encoding a ubiquitin-conjugating enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. *Proc. Natl. Acad. Sci. USA* **95**, 5678–5683 (1998).
- Hoffmann, R. M. & Pickart, C. M. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* **96**, 645–653 (1999).
- Urich, H. D. & Jentsch, S. Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. *EMBO J.* **19**, 3388–3397 (2000).
- Lawrence, C. W. The RAD6 DNA repair pathway in *Saccharomyces cerevisiae*: What does it do, and how does it do it? *Bioessays* **16**, 735–744 (1994).
- Ruffy, S., Laufer, S., Prakash, S. & Prakash, L. Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin-conjugating, DNA binding, and ATP hydrolytic activities. *J. Biol. Chem.* **272**, 23360–23365 (1997).
- Jentsch, S., McGrath, J. P. & Varshavsky, A. The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. *Nature* **328**, 131–134 (1987).
- Sung, P., Prakash, S. & Prakash, L. Mutation of cyclin-88 in the *Saccharomyces cerevisiae* RAD6 protein abolishes its ubiquitin-conjugating activity and its various biological functions. *Proc. Natl. Acad. Sci. USA* **87**, 2695–2699 (1990).
- Johnson, E. & Gupta, A. A. An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* **106**, 715–724 (2001).
- McDonald, J. P., Levine, A. S. & Woodgate, R. The *Saccharomyces cerevisiae* RAD30 gene, a homologue

of *Escherichia coli* *dmbB* and *smtC*, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. *Genetics* **147**, 1557–1568 (1997).

- Prakash, S. & Prakash, L. Translocation DNA synthesis in eukaryotes: A one- or two-polymerase affair. *Genes Dev.* **16**, 1872–1883 (2002).
- Haracka, L., Konradić, C. M., Urek, I., Prakash, S. & Prakash, L. Interaction with PCNA is essential for yeast DNA Polymerase ε function. *Mol. Cell* **8**, 407–415 (2001).
- Lawrence, C. W. & Maher, V. M. Mutagenesis in eukaryotes dependent on DNA polymerase zeta and rad18 mutations of *Saccharomyces cerevisiae*. *Mutat. Res.* **254**, 197–213 (1991).
- Spitzer, J., Safin, S., Haas, A. L. & Finley, D. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.* **15**, 1265–1273 (1995).
- Cassier-Chauvat, C. & Fabre, F. A similar defect in UV-induced mutagenesis conferred by the *rad6* and *rad18* mutations of *Saccharomyces cerevisiae*. *Mutat. Res.* **254**, 247–253 (1991).
- Lawrence, C. W. & Christensen, R. B. UV-mutagenesis in radiation-sensitive strains of yeast. *Genetics* **82**, 207–232 (1976).
- Hastings, P. J., Quah, S. K. & Von Borstel, R. C. Spontaneous mutation by mutagenic repair of spontaneous lesions. *Nature* **264**, 719–722 (1976).
- Morrison, A. et al. *REV1*, a *Saccharomyces cerevisiae* gene whose function is required for induced mutagenesis, is predicted to encode a non-essential DNA polymerase. *J. Bacteriol.* **171**, 5659–5667 (1989).
- Quah, S. K., Von Borstel, R. C. & Hastings, P. J. The origin of spontaneous mutation in *Saccharomyces cerevisiae*. *Genetics* **96**, 819–839 (1980).
- Brusky, J., Zhai, Y. & Xiao, W. *UBC73*, a DNA-damage-inducible gene, is a member of the error-free postreplication repair pathway in *Saccharomyces cerevisiae*. *Curr. Genet.* **32**, 168–174 (2000).
- Li, S.-Y. & Hochstrasser, M. A new protease required for cell-cycle progression in yeast. *Nature* **398**, 246–251 (1999).
- Li, S.-Y. & Hochstrasser, M. The yeast *ULP2* (*SMY4*) gene encodes a novel protease specific for the ubiquitin-like Sm3 protein. *Mol. Cell. Biol.* **20**, 2361–2377 (2000).
- Warbrick, E. The puzzle of PCNA many partners. *BioEssays* **22**, 997–1006 (2000).
- Lea, D. E. & Coulson, A. C. The distribution of the numbers of mutants in bacterial populations. *J. Genet.* **49**, 264–285 (1949).
- Von Borstel, R. C. et al. Topical reversion at the *HIS3* locus of *Saccharomyces cerevisiae*: a tale of three mutants. *Genetics* **148**, 1647–1654 (1998).

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Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan

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In diverse organisms, caloric restriction slows the pace of ageing and increases maximum lifespan. In the budding yeast *Saccharomyces cerevisiae*, caloric restriction extends lifespan by increasing the activity of Sir2 (ref. 1), a member of the conserved sirtuin family of NAD⁺-dependent protein deacetylases^{1–6}. Included in this family are SIR-2.1, a *Caenorhabditis elegans* enzyme that regulates lifespan⁷, and SIRT1, a human deacetylase that promotes cell survival by negatively regulating the p53 tumour

suppressor^{8–10}. Here we report the discovery of three classes of small molecules that activate sirtuins. We show that the potent activator resveratrol, a polyphenol found in red wine, lowers the Michaelis constant of SIRT1 for both the acetylated substrate and NAD⁺, and increases cell survival by stimulating SIRT1-dependent deacetylation of p53. In yeast, resveratrol mimics caloric restriction by stimulating Sir2, increasing DNA stability and extending lifespan by 70%. We discuss possible evolutionary origins of this phenomenon and suggest new lines of research into the therapeutic use of sirtuin activators.

Longevity regulatory genes have been identified in many eukaryotes, including rodents, flies, nematode worms and even single-celled organisms such as baker's yeast (reviewed in ref. 11). These genes seem to be part of an evolutionarily conserved pathway that evolved to promote survival in response to deteriorating environmental conditions. We recently reported that the pace of ageing in yeast is governed by *PNC1*, a caloric restriction-responsive and stress-responsive gene that depletes nicotinamide¹², a physiological inhibitor of Sir2¹³. On the basis of these results we proposed that caloric restriction might confer health benefits because it is a mild stress that provokes an organismal defence response¹².

On the basis of the positive health effects of caloric restriction in mammals¹⁴, we sought to identify small molecules that could modulate sirtuin activity. To this end, we screened a number of small molecule libraries, which included analogues of α -acetyl lysine, NAD⁺, NAD⁺ precursors, nucleotides and purinergic ligands. Fluorescent deacetylation assays were performed on human SIRT1 using a synthetic peptide substrate that encompassed lysine 382 (K382) of p53, a known target of SIRT1 *in vivo*^{8–10} and a preferred target *in vitro* (see Supplementary Fig. 1).

The initial compound screen identified several inhibitors of SIRT1 (Supplementary Table 7). However, the most notable outcome was the identification of two structurally similar compounds, quercetin and piceatannol, that stimulated SIRT1 activity five- and eightfold, respectively (Table 1). Both quercetin and piceatannol have previously been identified as protein kinase inhibitors^{15,16}. Comparison of the two compounds suggested a possible structure–activity relationship. The *trans*-stilbene ring structures of piceatannol are superimposable on the flavonol A and B rings of quercetin, with the ether oxygen and carbon-2 of the C ring aligning with the ethylene carbons in piceatannol (Table 1). Furthermore, the 5, 7, 3' and 4' hydroxyl group positions in quercetin can be aligned, respectively, with the 3, 5, 3' and 4' hydroxyls of piceatannol.

Both quercetin and piceatannol are polyphenols, members of a large and diverse group of plant secondary metabolites that include flavones, stilbenes, flavanones, isoflavones, catechins (flavan-3-ols), chalcones, tannins and anthocyanidins^{17,18}. A secondary screen of these compound families identified 15 additional SIRT1 activators that provided more than twofold stimulation (Table 1; see also Supplementary Tables 1, 2 and 4). We refer to the sirtuin-activating compounds as STACs.

Many, but not all of the most active STACs included hydroxyls in the two meta-positions of the A ring, *trans* to the B ring with a 4' or 3', 4' hydroxyl pattern (Table 1; see also Supplementary Tables 1 and 2). A potentially coplanar orientation and *trans*-positioning of the hydroxylated rings also appear to be important for activity. The most potent activator was resveratrol (3,5,4'-trihydroxystilbene), a polyphenol found in red wine that is associated with a surprising number of health benefits, most notably the mitigation of age-related diseases, including neurodegeneration, carcinogenesis and atherosclerosis^{17,19}.

The biological effects of polyphenols are frequently attributed to antioxidant, metal-ion-chelating and/or free-radical-scavenging activity¹⁷. We considered the possibility that the stimulation of SIRT1 might simply represent the repair of oxidative- or metal-ion-induced damage to the recombinant protein. We discounted this

explanation based on three observations. First, a variety of free-radical protective compounds, including antioxidants, chelators and radical scavengers failed to stimulate SIRT1 (see Supplementary Table 6). Second, among various polyphenols of equivalent anti-oxidant capacity²⁰ we observed diverse SIRT1-stimulating activity. Third, as described below, we showed that resveratrol could stimulate the activity of native sirtuins *in vivo*.

Dose-response experiments showed that resveratrol doubled the rate of deacetylation by SIRT1 at about 11 μ M and was saturated at 100–200 μ M (Fig. 1a). Although resveratrol had no significant effect on the two V_{max} determinations when either substrate or NAD⁺ was varied (Fig. 1b, c), it had pronounced effects on the two apparent Michaelis constants (K_m). Its effect on the acetylated peptide K_m was particularly striking, amounting to a 35-fold

Table 1 Stimulation of SIRT1 catalytic rate by plant polyphenols

Compound (100 μ M)	Ratio to control (mean \pm s.e.)	Structure
Resveratrol (3,5,4'-trihydroxy- <i>trans</i> -stilbene)	13.4 \pm 1.0	
Butenol (3,4,2',4'-tetrahydroxychalcone)	8.53 \pm 0.89	
Piceatannol (3,5,3',4'-tetrahydroxy- <i>trans</i> -stilbene)	7.90 \pm 0.50	
Isoquercitrin (4,2',4'-trihydroxychalcone)	7.57 \pm 0.84	
Fisetin (3,7,3',4'-tetrahydroxyflavone)	6.58 \pm 0.69	
Quercetin (3,5,7,3',4'-pentahydroxyflavone)	4.59 \pm 0.47	

Rate measurements with 25 μ M NAD⁺ and 25 μ M p53-382 acetylated peptide substrate were performed as described in Methods. All ratio data were calculated from experiments in which the total deacetylation in the control reaction was 0.25–1.25 μ M peptide or 1–5% of the initial concentration of acetylated peptide. s.e., standard error.

decrease (Fig. 1b). Resveratrol also lowered the K_m for NAD⁺ over fivefold (Fig. 1c). As resveratrol acts only on K_m , it could be classified as an allosteric effector of the 'K system' type²¹, which may indicate that only the substrate-binding affinity of the enzyme has been altered. In the presence of the negative regulator nicotinamide, resveratrol stimulated SIRT1, but there also appeared to be complex, concentration-dependent effects (Fig. 1d). Protein struc-

tural studies are underway to determine how resveratrol activates SIRT1 and to elucidate the interaction between the effects of nicotinamide and polyphenols.

To investigate the effects of sirtuin activators *in vivo*, we turned to *S. cerevisiae*. In this organism, a single extra copy of *SIR2* is sufficient to mimic calorie restriction¹. We determined that the optimal resveratrol concentration for activation of recombinant Sir2 was 2–5 μ M and that, in contrast to recombinant SIRT1, the rate stimulation was no more than about twofold (Fig. 2a). Resveratrol and other STACs in the stilbene, flavone and chalcone families were then tested for their effect on yeast replicative lifespan²². Three compounds—butein, fisetin and resveratrol—increased average lifespan by 31%, 55% and 70%, respectively, and all three significantly increased maximum lifespan (Fig. 2b). Higher concentrations of resveratrol provided no added lifespan benefit (Fig. 2c), and there was no lasting effect of the compound on the lifespan of pre-treated young cells (data not shown). Quercetin and piceatannol had no

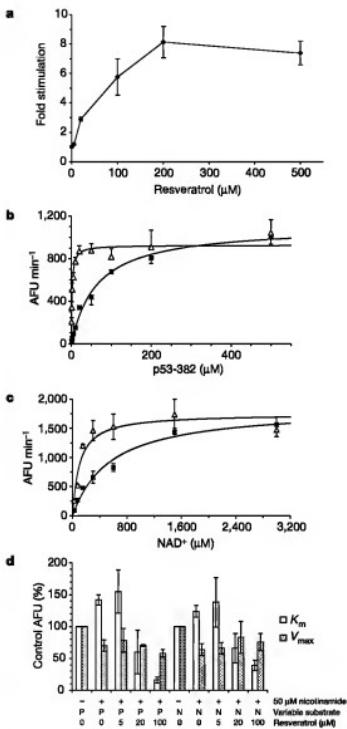


Figure 1 Effects of resveratrol on the kinetics of recombinant SIRT1. **a**, Resveratrol dose-response of SIRT1 catalytic rate. Fold stimulation values are derived from the means of six to nine determinations. **b**, SIRT1 initial rate at 3 mM NAD⁺, as a function of p53-382 acetylated peptide concentration in the presence (triangles) or absence (squares) of 100 μ M resveratrol. Lines represent nonlinear least square fits to the Michaelis-Menten equation. K_m (control, squares) = 64 μ M; K_m (plus resveratrol, triangles) = 1.6 μ M; V_{max} (control, squares) = 1,107 AUFl min⁻¹; V_{max} (plus resveratrol, triangles) = 926 AUFl min⁻¹. **c**, SIRT1 initial rate at 1 mM p53-382 acetylated peptide, as a function of NAD⁺ concentration, in the presence (triangles) or absence (squares) of 100 μ M resveratrol as in **b**. K_m (control, squares) = 550 μ M; K_m (plus resveratrol, triangles) = 101 μ M; V_{max} (control, squares) = 1,863 AUFl min⁻¹; V_{max} (plus resveratrol, triangles) = 1,749 AUFl min⁻¹. **d**, Kinetic constants are relative to untreated control and represent the mean of two determinations. AUFl, arbitrary fluorescence unit; N, NAD⁺; P, p53 acetylated peptide.

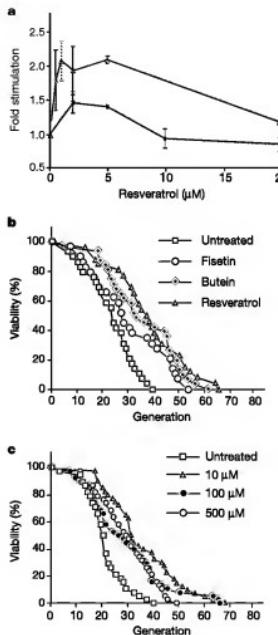


Figure 2 Effects of polyphenols on Sir2 and *S. cerevisiae* lifespan. **a**, Rates were determined using either Fc⁺ acetylated lysine substrate (squares) or an acetylated fluorogenic peptide (triangles) based on the sequence encompassing histone H4 Lys 16, a target of Sir2 *in vivo* (Supplementary Fig. 1). Fold stimulations represent the mean of at least three determinations. **b**, Replicative lifespan is the number of daughter cells an individual mother cell produces before dying. Lifespan analyses were determined by micro-manipulating individual yeast cells²⁰ on complete 2% glucose medium with 10 μ M compound, unless otherwise stated. Average lifespan for wild type, 29.2 generations; fisetin, 30.0; butein, 35.5; resveratrol, 36.8. **c**, Average lifespan for wild type untreated, 21.0 generations; grown on resveratrol 10 μ M, 35.7; 100 μ M, 29.4; 500 μ M, 29.3.

significant effect on lifespan (data not shown), possibly due to oxidation of the compounds in the medium or insufficient uptake into cells.

For subsequent experiments we focused on resveratrol. Glucose restriction, a form of calorie restriction in yeast, resulted in no significant extension of long-lived, resveratrol-treated cells (Fig. 3a), indicating that resveratrol probably acts through the same pathway as calorie restriction. Consistent with this, resveratrol had no effect on the lifespan of a *sir2* null mutant (Fig. 3b). Given that resveratrol is reported to have fungicidal properties at high concentrations and that mild stress can extend yeast lifespan by activating *PNC1* (ref. 12), it was plausible that resveratrol was extending lifespan by inducing *PNC1*, rather than acting on *Sir2* directly. However, resveratrol extended the lifespan of a *pnc1* null mutant nearly as well as it did wild-type cells (Fig. 3b). Together these data show that resveratrol acts downstream of *PNC1* and requires *SIR2* for its effect. Although we do not discount the possibility that resveratrol might act on *Sir2* indirectly, the simplest explanation of our observations is that resveratrol increases lifespan by directly stimulating *Sir2* activity *in vivo*.

The ability of *Sir2* to extend yeast lifespan is thought to stem from its role in stabilizing repetitive DNA^{12–24}. Homologous recombination between ribosomal DNA (rDNA) repeats can generate an extrachromosomal circular DNA molecule that is replicated until it reaches toxic levels in old cells²³. Consistent with this, resveratrol reduced the frequency of rDNA recombination by about 60% in a *SIR2*-dependent manner (Fig. 3c, d). In the presence of nicotinamide, recombination was also decreased by resveratrol, in agreement with the kinetic data (Fig. 3c). Interestingly, each of the STACs we tested had only minor effects on rDNA silencing compared with the 2×*SIR2* positive control (Fig. 3e, f). Together, these findings add to the body of evidence that yeast ageing is caused by genomic instability^{1,22–25}, not gene dysregulation²⁶.

Another measure of lifespan in *S. cerevisiae* is the length of time

that cells can survive in a metabolically active but nutrient-deprived state. Ageing under these conditions (that is, chronological ageing) is primarily due to oxidative damage. Resveratrol (10 μM or 100 μM) failed to extend chronological lifespan (not shown), indicating that the effect of resveratrol on sirtuins may be more relevant than its antioxidant activity^{17,18}.

To test whether STACs could stimulate human sirtuins *in vivo*, we developed a cell-based fluorescence deacetylase assay (Supplementary Figs 2 and 3a–c). A selection of sirtuin-stimulatory and non-stimulatory polyphenols were analysed using this *in vivo* assay and values were plotted against their fold stimulation of SIRT1 *in vitro* (Fig. 4a). Compounds with little or no activity *in vitro* clustered around the negative control (group A). Another grouping of strong *in vitro* activators is clearly distanced from the low-activity cluster in both dimensions (group B). These data indicate that certain polyphenols can activate human sirtuins *in vivo*.

Next, we assessed whether resveratrol could activate SIRT1 *in vivo*. One known target of SIRT1 is lysine 382 of p53 (K382). Deacetylation of this residue by SIRT1 decreases the activity and half-life of p53, and increases cell survival under a variety of DNA-damaging conditions^{8–10}. Treatment of cells with a low concentration (0.5 μM) of resveratrol increased cell survival after ionizing irradiation (Fig. 4b). To test whether this was due to modulation of SIRT1 activity, we generated an antibody that specifically recognizes the acetylated form of p53-K382 (Ac-K382) (Fig. 4c and data not shown). U2OS cells treated with 0.5 μM resveratrol showed a marked decrease (about 75%) in the level of Ac-K382 (Fig. 4c; see also Supplementary Fig. 4a). At higher concentrations of resveratrol (>50 μM) the effect was reversed (Fig. 4d and data not shown), which may explain the dichotomy in the literature regarding the effects of resveratrol on cell viability^{17,27,28}. Treatment of HEK 293 cells with resveratrol led to a dose-dependent decrease in Ac-K382 levels that was diminished by overexpression of a dominant-negative SIRT1 allele (see Fig. 4e and Supplementary Fig. 4b).

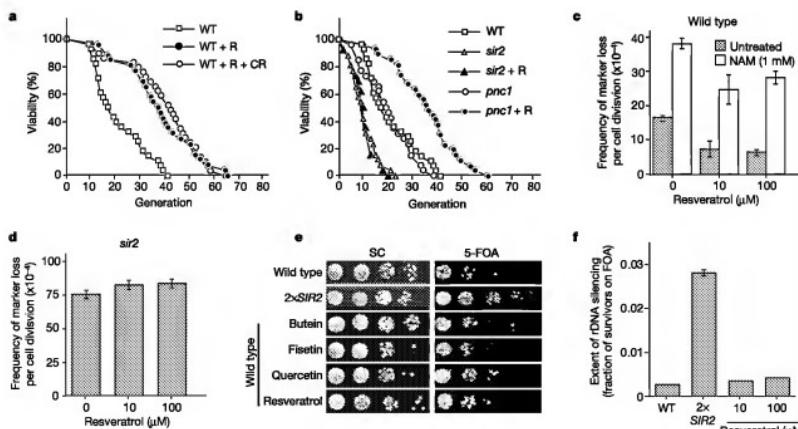


Figure 3 Resveratrol extends lifespan by mimicking caloric restriction and suppressing rDNA recombination. **a**, Average lifespan for WT (untreated, 19.0 generations; wild type plus resveratrol (WT + R), 37.8; glucose (calorie)-restricted plus resveratrol (WT + CR + R), 39.9. **b**, Average lifespan for wild type, 19.0 generations; *sir2*, 9.9; *sir2* plus resveratrol, 10.0; *pnc1*, 19.2; *pnc1* plus resveratrol, 33.1. **c, d**, Recombination frequencies for wild type and *sir2* strains were determined by monitoring loss of the rDNA::ADE2 marker gene. **e**, Pre-treated *RDY11*:URA3 cells were spotted as tenfold serial dilutions on SC medium with or without 5-fluoroorotic acid (5-FOA). Increased silencing results in increased survival on 5-FOA medium. **f**, Level of rDNA silencing represents numbers of surviving cells on 5-FOA/total plated.

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These data strongly indicate that resveratrol can promote cell survival by stimulating SIRT1 *in vivo*.

We have discovered small molecule sirtuin activators (STACs), and have shown that they can promote the survival of eukaryotic cells. Sirtuins have been found in diverse eukaryotes including fungi, protozoans, metazoans and plants²⁹, and probably evolved early in life's history³⁰. Plants produce a variety of polyphenols such as resveratrol in response to stresses including dehydration, nutrient deprivation, ultraviolet radiation and pathogens³⁰. Therefore it is plausible that plants synthesize these molecules in order to regulate a sirtuin-mediated stress response. This would be consistent with

the recently discovered relationship between environmental stress and Sir2 activity in yeast¹². Perhaps these compounds have stimulatory activity on sirtuins from fungi and animals because they mimic an endogenous activator. Alternatively, animal and fungal sirtuins may have retained or developed an ability to respond to plant stress metabolites because they are a useful indicator of a deteriorating environment and/or food supply. Consistent with these ideas, preliminary experiments indicate that resveratrol can also extend the lifespan of multicellular animals, including the nematode *C. elegans* and the fruitfly *Drosophila melanogaster* (J.G.W., D.A.S. and M. Tatar, unpublished results).

A broad range of human health benefits have been reported for plant polyphenols including cardioprotection, neuroprotection and cancer suppression^{17–19}. Interestingly, similar beneficial effects are observed for calorie-restricted rodents³¹. We postulate that many of the effects of polyphenols might be the result of a calorie-restriction-mimetic defence response mediated by sirtuins. Part of this response may involve suppressing p53 and delaying apoptosis to give cells additional time to repair damage and to prevent unnecessary cell death. Our results also suggest that survival and longevity at the cellular and organismal levels are intimately linked. The newly appreciated ability of polyphenols to promote survival and longevity by activating sirtuins indicates a new line of investigation into the effects of these and related molecules on age-related human diseases. □

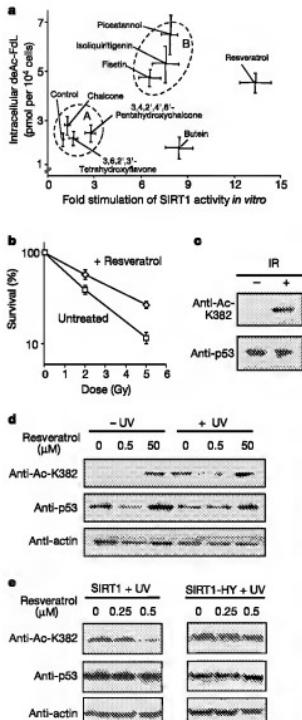


Figure 4 STACs stimulate sirtuin activity in human cells. **a**, STACs can stimulate TSA insensitive FDL. deacetylation (Supplementary Figs 2 and 3). Intracellular deacetylated FDL (deAc-FDL) levels are plotted against fold stimulation in the *in vitro* SIRT1 assay. **b**, Survival of HEK 293 cells in the presence of 0 (squares) or 0.5 μM (diamonds) resveratrol after exposure to 0, 2 or 5 Gy of ionizing radiation (IR). **c**, U2OS osteosarcoma cells were exposed to 0 or 10 Gy of ionizing radiation (IR). Cell lysates were prepared 4 h after irradiation and probed with indicated antibodies. **d**, U2OS cells were pre-treated with resveratrol for 4 h after which cells were exposed to 0 or 50 μM cm^{-2} of ultraviolet (UV) radiation. **e**, HEK 293 cells expressing wild-type SIRT1 or dominant negative SIRT1 H363Y (SIRT1-HY) were pre-treated with resveratrol and exposed to ultraviolet radiation as above.

Methods

Compound libraries and deacetylation assays

His₆-tagged recombinant SIRT1 and recombinant Sir2 were prepared as described¹². A total of 0.1–1.0 μg of SIRT1 and 1.5 μg of Sir2 were used per deacetylation assay (50 μl total reaction) using 'Flour de lys' (FIL) as described¹². SIRT1 screening assays used the p53-382 acetylated substrate (FIL-SIRT1, BIOMOL). Themed compound libraries (BIOMOL) were used for primary and secondary screening. Most polyphenol compounds were dissolved at 10 mM in dimethylsulfoxide (DMSO) on the day of the assay. For water soluble compounds and negative controls, 1% v/v DMSO was added to the assay. *In vitro* fluorescence assays were read in white, half volume 96-well microplates (Corning) with a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, excitation 360 nm, emission 460 nm, gain = 85). HeLa cells were grown and the cellular deacetylation assays performed and read, as above, in full-volume 96-well microplates (Corning). Unless otherwise indicated all initial rate measurements were means of three or more replicates, obtained with single-incubation times, at which point 5% or less of the substrate initially present had been deacetylated.

For the SIRT1 resveratrol dose-response experiments, values are derived from the slopes of fluorescence (arbitrary fluorescence units, AFU) against time plots with data obtained at 0, 5, 10 and 20 min of deacetylation using 25 μM each of NAD⁺ and p53-382 acetylated peptide. Sir2 assays were performed similarly using either 100 nM FDL or 25 μM acetylated H4 peptide (FIL-H4-Ac-K16). Calculation of net fluorescence included the subtraction of a blank value by adding an inhibitor (200 μM sirtuin or 1 mM nicotinamide) to the reaction in the case of SIRT1, and in the case of Sir2 by omitting the enzyme from the reaction. For polyphenols that partially quenched the fluorescence produced in the assay, correction factors were obtained by determining the fluorescence increase due to a 3 μM spike of an FDL deacetylated standard (BIOMOL).

Medium and strains

All yeast strains were grown at 30 °C in complete yeast extract/bactopeptone, 2.0% (w/v) glucose (YPD) medium unless stated otherwise. Calorie restriction was induced in 0.5% glucose. Synthetic complete (SC) medium consisted of 1.67% yeast nitrogen base, 2% glucose and 40 mM⁻¹ each of aromatic markers. Sir2 was integrated in extra copy and disrupted as described¹². Other strains are described elsewhere¹². For cellular deacetylation assays, HeLa S3 cells were used. U2OS osteosarcoma and human embryonic kidney (HEK 293) cells were cultured adherently in DMEM containing 10% fetal calf serum with 1.0% glutamine and 1.0% penicillin/streptomycin. HEK 293 overexpressing dominant-negative SIRT1 H363Y was a gift of R. Frye.

Lifespan determinations

Lifespan measurements were performed using PSV316AT MAT6 as previously described¹². All compounds for lifespan analyses were dissolved in 95% ethanol. Plates were dried and used with 24 h. Plates were pre-incubated on their respective medium for at least 15 h and equilibrated on the medium for a minimum of 4 h before micro-manipulating them. At least 30 cells were examined per experiment and each experiment was performed at least twice. Statistical significance of lifespan differences was determined using the Wilcoxon rank sum test. Differences are stated to be significant when the confidence is higher than 95%.

Silencing and recombination assays

Ribosomal DNA silencing assays were performed as previously described¹. Ribosomal DNA recombination frequencies were determined by plating W303AR cells¹³ on YPD medium with low adenine/histidine and counting the fraction of red sector colonies using Bio-Rad Quantity One software. At least 6,000 cells were analysed per experiment and all experiments were performed in triplicate. All strains were pre-grown for 15 h with the relevant compound before plating.

Cellular survival assay

HEK 293 cells treated with either 0.5 μM resveratrol or 0.5% DMSO for 8 h were trypsinized, collected and exposed to 0, 2 or 5 Gy of ionizing radiation. A total of 300 cells per sample were then plated in duplicate in 10 cm dishes. Medium was changed the following day and after 10 days, colonies were stained with crystal violet and counted. Error bars represent the standard error of the mean.

Proteins and western analyses

Recombinant Sir2-glutathione S-transferase was expressed and purified from *Escherichia coli* as previously described except that lysates were prepared using sonication¹⁴. Recombinant SIRT1 from *E. coli* was prepared as described¹. Polyclonal antiserum against p53-K382 was generated using an acetylated peptide antigen as previously described¹⁵, with the following modifications. Anti-Ac-K382 antibody was affinity purified using non-acetylated p53-K382 peptide and stored in PBS at -70 °C. Western hybridizations using anti-acetylated K382 or anti-acin (Chemicon) antibody were performed at 1:1,000 dilution of antibody. Hybridizations with polyclonal p53 antibody (Santa Cruz) used a 1:500 dilution. Cell extracts were prepared 4 h after irradiation from cells pre-treated with resveratrol for 4 h. Lysis buffer contained 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, 1% NP40, 1 mM dithiothreitol and anti-protease cocktail (Roche).

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- Liu, S. J., DeFesa, F. A. & Guarente, L. Requirement of NAD and SIR2 for life span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science* **289**, 2126–2128 (2000).
- Lansley, J. et al. The silencing protein Sir2 and its homologs are NAD-dependent protein deacetylases. *Proc. Natl. Acad. Sci. USA* **97**, 5807–5811 (2000).
- Irnar, S., Armstrong, C. M., Kaeberlein, M. & Guarente, L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795–800 (2000).
- Smith, J. S. et al. A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. *Proc. Natl. Acad. Sci. USA* **97**, 6658–6663 (2000).
- Tanter, K. G., Lansley, J., Stargelana, R. & DeRisi, J. M. Silent information regulator 2 family of NAD-dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. *Proc. Natl. Acad. Sci. USA* **97**, 14778–14782 (2000).
- Tanay, J. C., Dowd, C. J., Huang, J., Hiltz, H. & Meozed, D. An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. *Cell* **99**, 735–745 (1999).
- Tissenbaum, H. A. & Guarente, L. Increased dosage of a sir-2 gene extends lifespan in *C. elegans*. *Nature* **410**, 227–230 (2001).
- Vashisht, H. et al. ISR1(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107**, 149–159 (2001).
- Luo, J. et al. Negative control of p53 by Sir2α promotes cell survival under stress. *Cell* **107**, 157–148 (2001).
- Langley, E. P. M. et al. Human Sir2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *EMBO J.* **21**, 2385–2394 (2002).
- Keryan, C. A conserved regulatory mechanism for aging. *Cell* **105**, 165–168 (2001).
- Anderman, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O. & Sinclair, D. A. Nicotinamide and Pnc1 govern lifespan extension by calorie restriction in *S. cerevisiae*. *Nature* **423**, 183–185 (2003).
- Bitterman, K. J., Anderson, R. M., Cohen, H. Y., Laaber-Etterbeek, M. & Sinclair, D. A. Inhibition of silencing and accelerated ageing by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J. Biol. Chem.* **277**, 4599–45107 (2002).
- Masere, E. J. Calorie restriction and aging: an update. *Exp. Gerontol.* **35**, 295–305 (2000).
- Glossmann, H., Probst, P. & Eggeroth, E. Quercetin inhibits tyrosine phosphorylation by the cyclic nucleotide-independent transforming protein kinase, pp60^{src}. *Naunyn Schmiedebergs Arch. Pharmacol.* **317**, 100–102 (1981).
- Oliver, J. M., Bung, D. L., Wilson, B. S., McLaughlin, J. L. & Geblek, R. L. Inhibition of yeast cell cycle protein I-mediated signalling and effector function by the S-type-selective inhibitor, pisetastatin. *J. Biol. Chem.* **269**, 2969–29703 (1994).
- Ferguson, J. R. Role of plant polyphenols in genomic stability. *Mutat. Res.* **475**, 89–111 (2001).
- Middleton, E. J., Kandaswami, C. & Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **52**, 673–751 (2000).
- Jang, M. et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**, 218–220 (1997).
- Stojanovic, S., Spritz, H. & Brode, O. Efficiency and mechanism of the antioxidant action of resveratrol and its analogues in the radical liposome oxidation. *Arch. Biochem. Biophys.* **391**, 79–89 (2001).
- Monod, J., Wyman, J. & Changenx, J. P. On the nature of allosteric transitions. *J. Mol. Biol.* **12**, 88–118 (1965).
- Sinclair, D. A. Paradigms and pitfalls of yeast longevity research. *Mech. Ageing Dev.* **123**, 857–867 (2002).
- Sinclair, D. A. & Guarente, L. Extrachromosomal rRNA circles: a cause of aging in yeast. *Cell* **91**, 1033–1042 (1997).
- DeFesa, F. A. et al. Elimination of replication block protein Fob1 extends the life span of yeast mother cells. *Mol. Cell* **3**, 447–455 (1998).
- Kaeberlein, M., McVey, M. & Guarente, L. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev.* **13**, 2570–2580 (1999).
- Jaworski, S. M. Metabolic control and gene dysregulation in yeast aging. *Ann. NY Acad. Sci.* **908**, 21–30 (2000).

- Zhang, Z. Molecular mechanism of the chemopreventive effect of resveratrol. *Mutat. Res.* **523**–534, 145–150 (2003).
- Nicolinis, G., Rigolos, R., Milos, M., Bettella, A. A. & Trofici, G. Anti-apoptotic effect of trans-resveratrol on puchitinduced apoptosis in the human neuroblastoma SH-SY5Y cell line. *Neurosci. Lett.* **362**, 41–44 (2004).
- Pandey, A. et al. Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res.* **30**, 5036–5055 (2002).
- Soleas, G. J., Dismanidis, E. P. & Goldberg, D. M. Resveratrol, a molecule whose time has come? *And. genet. Clin. Biochem.* **30**, 91–113 (1997).

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A cell surface receptor mediates extracellular Ca^{2+} sensing in guard cells

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Extracellular Ca^{2+} ($\text{Ca}_{\text{o}}^{2+}$) is required for various physiological and developmental processes in animals and plants^{1–3}. In response to varied $\text{Ca}_{\text{o}}^{2+}$ levels, plants maintain relatively constant internal Ca^{2+} content, suggesting a precise regulatory mechanism for Ca^{2+} homeostasis⁴. However, little is known about how plants monitor $\text{Ca}_{\text{o}}^{2+}$ status and whether $\text{Ca}_{\text{o}}^{2+}$ -sensing receptors exist. The effects of $\text{Ca}_{\text{o}}^{2+}$ on guard cells in promoting stomatal closure by inducing increases in the concentration of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{c}}$)^{5–8} provide a clue to $\text{Ca}_{\text{o}}^{2+}$ sensing. Here we have used a functional screening assay in mammalian cells⁹ to isolate an *Arabidopsis* complementary DNA clone encoding a Ca^{2+} -sensing receptor, CAS. CAS is localized to the plasma membrane, exhibits low-affinity/high-capacity Ca^{2+} binding, and mediates Ca^{2+} -induced $[\text{Ca}^{2+}]_{\text{c}}$ increases. CAS is expressed predominantly in the shoot, including guard cells. Repression of CAS disrupts $\text{Ca}_{\text{o}}^{2+}$ signalling in guard cells, and impairs bolting (swift upward growth at the transition to seed production) in response to Ca^{2+} deficiency, so we conclude that CAS may be a primary transducer of $\text{Ca}_{\text{o}}^{2+}$ in plants.

In plants, a high proportion of the total Ca^{2+} is often located in the cell wall and at the exterior surface of the plasma membrane^{9–10}. $\text{Ca}_{\text{o}}^{2+}$ is important for the stability of the wall and the membrane, and is essential in many physiological processes, such as root-tip growth, pollen-tube elongation, light perception, and phytohormone action¹¹. In contrast to cytosolic Ca^{2+} sensing¹¹, how plants perceive $\text{Ca}_{\text{o}}^{2+}$ and coordinate their growth and development with the status of $\text{Ca}_{\text{o}}^{2+}$ remains largely unknown. It has long been established that $\text{Ca}_{\text{o}}^{2+}$ promotes stomatal closure^{5,12} by triggering